PATENT

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:

KLIMPEL *et al.*

Application No.: 08/937,276

Filed: September 15, 1997

For: TARGETING ANTIGENS TO THE MHC CLASS I PROCESSING PATHWAY WITH AN ANTHRAX TOXIN FUSION PROTEIN

Examiner: Schwadron, Ronald B.

Art Unit: 1644

COMMUNICATION UNDER

37 C.F.R. §§ 1.821-1.825

AND

AMENDMENT

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Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

In response to the Notice to Comply with Requirements for Patent Applications Containing Nucleotide Sequence and/or Amino Acid Sequence Disclosures, 37 C.F.R. §§ 1.821-1.825, that accompanied the Examiner's Communication mailed November 23, 2001, Applicants submit herewith the required paper copy and computer readable copy of the Substitute Sequence Listing. Please amend the specification in adherence with 37 C.F.R. §§ 1.821-1.825 as follows.

EXHIBIT

A

In the Specification:

Please replace the paragraph beginning at page 25, line 3 with the following:

--The LF-gp120 fusion protein and PA were tested *in vitro* for their ability to deliver antigenic proteins to the cell cytosol for processing and presentation with MHC class I molecules on the cell surface. Mouse mastocytoma cells were used as the target antigen-presenting cell. Cytotoxic T lymphocytes that recognized the peptide epitope RGPGRAFVTI (SEQ ID NO:5) from the V3 loop of gp120 were used with a ^{51}Cr-release assay to examine specific lysis of the antigen presenting target cell population. Translocation-deficient mutant PA proteins or the absence of PA were used as controls to demonstrate that processing of the fusion protein relies on internalization via the PA receptor.--

Please replace the paragraph beginning at page 25, line 13 with the following:

--The following cell lines were used in this example. P815, a DBA/2 derived (H-2^d) mastocytoma (ATCC TIB-64) was used as target cells in the cytotoxic T lymphocyte (CTL) assay. These cells were maintained in RPMI1640 supplemented with 10% FCS. The HIV gp120-specific CTL line 9.23.3 that recognizes the V3 epitope RGPGRAFVTI (SEQ ID NO:5) that has been previously described (Takahashi et al., *Proc. Natl. Acad. Sci. U.S.A.* 85: 3105-3109 (1988); Alexander-Miller et al., *Proc. Natl. Acad. Sci. U.S.A.* 93: 4102-4107 (1996); Shirai et al., *J Immunol.* 148: 1657-1667 (1992)). Peptide P18IIIB that recognizes this epitope was made by an automated peptide synthesizer (Applied Biosystems) and purified by high performance liquid chromatography before use. The HIV gp120-specific CTL line was derived from BALB/C spleens taken from mice previously immunized with a recombinant vaccinia

virus expressing the gp120 protein. 9.23.3 CTL were stimulated with 10 μm free P18IIIB peptide at 5 x 10^5 CTL, and 5 x 10^6 irradiated spleenocytes [3000 rads (1 rad = 0.01 Gy)] per well, in a 24-well plate containing 2 ml of a 1:1 mixture of RPMI1640 medium and Eagle-Hank's amino acid medium (EHAA) supplemented with L-glutamine, sodium pyruvate, nonessential amino acids, penicillin, streptomycin, 5 x 10^{-5} M 2-mercaptoethanol, 10% fetal calf serum, and 10% T-stim (Collaborative Biomedical Products).--

Please cancel the present "SEQUENCE LISTING", pages 1-3, and insert therefor the accompanying paper copy of the Substitute Sequence Listing, page numbers 1 to 3, at the end of the application.

REMARKS

The amendment to the paragraph beginning on line 3 of page 25 changes the sequence of the peptide epitope from the V3 loop of HIV gp120 from an incorrect sequence, RGPGRRAFNTI, to the correct sequence, RGPGRAFVTI, also found at page 25, line 17, also described as the "V3 epitope" of HIV gp120. Lines 17-18 also provide the Takahashi et al., *Proc. Natl. Acad Sci. U.S.A.* 85: 3105-3109 (1988) reference describing this epitope peptide, which isenclosed herein for the convenience of the Examiner. On page 3107, column 1, lines 16-17, of thais reference, a peptide sequence containing the correct sequence for SEQ ID NO:5 is found. Thus, the correction of this sequence does not introduce new matter. This corrected sequence has been incorporated into the Substitute Sequence Listing as SEQ ID NO:5.

Applicants request entry of this amendment in adherence with 37 C.F.R. §§1.821 to 1.825. This amendment is accompanied by a floppy disk containing the above named sequences, SEQ ID NOS:1-5, in computer readable form, and a paper copy of the sequence information which has been printed from the floppy disk.

The information contained in the computer readable disk was prepared through the use of the software program "PatentIn" and is identical to that of the paper copy.

Attached hereto is a marked-up version of the changes made to the Specification by the current Amendment. The attached pages are captioned "**VERSION WITH MARKINGS TO SHOW CHANGES MADE**".

If the Examiner believes a telephone conference would expedite prosecution of this application, please telephone the undersigned at 415-576-0200.

Respectfully submitted,

Annette S. Parent
Reg. No. 42,058

TOWNSEND and TOWNSEND and CREW LLP
Two Embarcadero Center, 8th Floor
San Francisco, California 94111-3834
Tel: (415) 576-0200
Fax: (415) 576-0300
ASP:dmw

VERSION WITH MARKINGS TO SHOW CHANGES MADE

In the Specification:

Paragraph beginning at line 3 of page 25 has been amended as follows:

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PRIOR ART SEARCHES, INC.

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U.S.A.
email: tommyf@priorart.com
http://www.priorart.com

Fax
800-369-1424
703-521-6503

December 18, 2001

Karen J. Iovino
Townsend and Townsend
and Crew, LLP
8th Floor
Two Embarcadero Center
San Francisco, CA 94111-3834

RE: HAND DELIVERY
S/N 08/937,276
YOUR REF: 015280-290100US
PAS NO: 01-17492

Dear Karen:

As authorized by your FedEx letter of December 17, 2001, the materials related to the above application has been hand carried to Group 1600 of the USPTO on December 17, 2001.

Kindly find enclosed your return postcard that has been date-stamped by the Receptionist of Group 1600 to indicate receipt. Also, the signed Certificate of Delivery sheets are enclosed.

Kindly call if you have a question.

Very truly yours,

PRIOR ART SEARCHES, INC.

Tommy L. Foster

TLF:htv:0117492R
Enclosures

Proc. Natl. Acad. Sci. USA
Vol. 85, pp. 3105–3109, May 1988
Immunology

EXHIBIT
B

An immunodominant epitope of the human immunodeficiency virus envelope glycoprotein gp160 recognized by class I major histocompatibility complex molecule-restricted murine cytotoxic T lymphocytes

(acquired immunodeficiency syndrome/immune-response genes/peptides/vaccine/amphipathicity)

HIDEMI TAKAHASHI[a], JOSEPH COHEN[b,c], ANNE HOSMALIN[a], KEMP B. CEASE[a,d], RICHARD HOUGHTEN[e], JAMES L. CORNETTE[f], CHARLES DELISI[g], BERNARD MOSS[h], RONALD N. GERMAIN[b], AND JAY A. BERZOFSKY[a,i]

[a]Metabolism Branch, National Cancer Institute, and [b]Laboratory of Immunology and [h]Laboratory of Viral Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892; [c]Department of Molecular Biology, Scripps Clinic and Research Foundation, La Jolla, CA 92037; [f]Department of Mathematics, Iowa State University, Ames, IA 50011; and [g]Department of Biomathematical Sciences, Mt. Sinai Medical School, New York, NY 10029

Contributed by Bernard Moss, December 24, 1987

ABSTRACT **Because cytotoxic T lymphocytes (CTL) may be important for preventing direct cell-to-cell transmission of human immunodeficiency virus (HIV), the agent responsible for acquired immunodeficiency syndrome, we have begun to investigate the epitope specificity and immune response (*Ir*) gene control of anti-HIV CTL responses in experimental animals. Mice were infected with a recombinant vaccinia virus expressing the HIV gp160 envelope gene, and the primed lymphocytes were restimulated *in vitro* with a transfected histocompatible cell line expressing the same gene. Our results show that H-2^d mice are CTL high responders and H-2^k mice are low responders to the HIV gp160 envelope protein under these conditions. Moreover, the H-2^d mice respond predominantly to a single immunodominant site represented by a 15-residue synthetic peptide conforming to the amphipathic α-helix model of T-cell epitopes and seen by CD4$^-$ CD8$^+$ CTL in association with the D^d class I major histocompatibility complex (MHC) molecules. The facts that CTL responses were detected in the context of only one of four class I MHC molecules tested and that the response was limited predominantly to a single epitope indicate that the CTL repertoire elicited by the HIV envelope protein in association with murine class I MHC molecules may be very limited. In addition, this epitope occurs in a highly variable segment of the envelope protein. This puts constraints on the use of a single peptide sequence from this region in a vaccine, as such a vaccine would have to be polyvalent. Nevertheless, this same variability suggests that this region may be under selective pressure from human CTL, and therefore that this site may be immunodominant in humans as well as mice and so of clinical importance in vaccine development.**

Immunological responses to viral infection include both humoral and cell-mediated effector mechanisms. For most viruses, vaccine design has stressed elicitation of neutralizing antibodies, although T-cell-mediated immunity, particularly involving class I major histocompatibility complex (MHC) molecule-restricted cytotoxic T lymphocytes (CTL), can play an important role in resistance to viral infection (1). To date, attempts to deal with infection by human immunodeficiency virus 1 (HIV-1), the agent of acquired immunodeficiency syndrome (AIDS) (2–4), have focused on drug therapy of infected individuals (5) and candidate vaccines able to elicit neutralizing antibodies against the gp160 envelope glycoprotein of this virus (6–11). However, HIV can be transmitted via infected cells and spread by passage between fused cells without a requirement for extracellular virus accessible to antibody (12). Thus, a vaccine strategy that includes elicitation of specific CTL effectors able to kill cells producing HIV proteins may be particularly useful in preventing successful infection and/or virus spread. Evidence for circulating CTL in HIV-infected individuals has been obtained (13, 14). The envelope and to a lesser degree the internal gag proteins were identified as targets. However, no specific epitopes recognized by CTL within any of the HIV proteins have yet been identified.

Our original studies on HIV immunogenicity concentrated on epitopes able to stimulate class II MHC molecule-restricted T cells involved as helper cells in humoral immune responses. By use of an algorithm for identifying regions of proteins that were likely to serve as helper T-cell immunogens due to an ability to assume an amphipathic α-helical structure (15–17), several short gp160 peptides that could effectively stimulate T-helper cell immunity in mice were identified (18). Recently, class I MHC molecule-restricted CTL also have been shown to recognize short peptide fragments of native proteins (19–21), as originally demonstrated for class II MHC molecule-restricted helper T cells (22). To explore the specificity of CTL responses to HIV peptide antigens and the role of MHC-linked immune response genes in such responses, we have developed a model system for eliciting immunity to cloned gene segments involving *in vivo* immunization with recombinant vaccinia vectors and *in vitro* stimulation with syngeneic transfected cells expressing the protein of interest. Using this protocol, we demonstrate significant CTL responses to the gp160 glycoprotein in H-2^d, but not in H-2^k, mice. The effector cells are CD4$^-$ CD8$^+$ and restricted to the class I MHC molecule D^d. Analysis of 41 overlapping peptides from the gp160 sequence revealed that a single peptide fragment in the gp160 envelope protein appears to be immunodominant for CTL recognition in responder H-2^d mice. The location of this major epitope in a region of interisolate variation suggests that the activity of human immune effector mechanisms directed against this region of the molecule leads to selection

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Abbreviations: HIV, human immunodeficiency virus; CTL, cytotoxic T lymphocyte(s); MHC, major histocompatibility complex; AIDS, acquired immunodeficiency syndrome.

[c]Present address: Albany Medical College, Albany, NY 12208.
[d]Present address: Department of Internal Medicine, University of Michigan Medical School, Ann Arbor, MI 48109.
[i]To whom reprint requests should be addressed.

of variant viruses. This result also puts limitations on the use of a single peptide sequence from this region as a component of a general anti-HIV vaccine.

MATERIALS AND METHODS

Mice. BALB/c (H-2^d) and C3H/HeN (H-2^k) mice were obtained from The Jackson Laboratories. BALB.K (H-2^k) and BALB/c mice used in certain experiments were bred in our own colony. Mice used were 6–12 weeks old.

Recombinant Vaccinia Viruses. vSC-8 (recombinant vaccinia vector containing the bacterial *lacZ* gene), and vSC-25 (recombinant vaccinia vector expressing the HIV env glycoprotein gp160 of the HTLV III_B isolate without other HIV structural or regulatory proteins) have been described (23).

Transfectants. An *Eco*RV fragment containing the region of HIV clone BH.8 encoding gp160 (24) used to construct vSC-25 was cloned into the simian virus 40-driven expression vector pcEXV-3 (25). This DNA was cotransfected along with pSV2neo into either BALB/c 3T3 (H-2^d) or DAP.3 L-cell (H-2^k) fibroblasts by a standard calcium phosphate precipitation method (26). Transfectants were also prepared with pSV2neo alone. Individual clones were picked after selection in G418 (Geneticin, GIBCO), expanded, and tested for expression of the gp160 gene by RNA hybridization with the *Eco*RV fragment labeled by random hexamer priming as probe. T1.1.1 and T4.8.3 are L-cell transfectants expressing the L^d and D^d class I MHC molecules, respectively (26, 27), and were generously provided by David Margulies (National Institute of Allergy and Infectious Diseases).

Monoclonal Antibodies. The following monoclonal antibodies were used: Anti-Lyt2 (3.155; rat IgM) (28) and anti-L3T4 (RL172.4; rat IgM) (29).

CTL Generation. Mice were immunized i.v. with 10^7 plaque-forming units of recombinant vaccinia viruses. Three to 14 wk later, immune spleen cells [5×10^6 cells per ml in 24-well culture plates in complete T-cell medium (18)] were restimulated for 6 days *in vitro* with either recombinant vaccinia virus-infected syngeneic spleen cells (2.5×10^6 cells per ml) (1 hr, 37°C, multiplicity of infection, 10:1) or gp160 gene-transfected MHC-identical fibroblasts (2×10^5 cells per ml). A long-term CTL line was also generated by repetitive stimulation of immune cells with mitomycin-treated gp160 gene-transfected fibroblasts and 10% Con A supernatant-containing medium.

CTL Assay. After culture for 6 days, cytolytic activity of the restimulated cells was measured as described (30) by using a 6-hr assay with various ^{51}Cr-labeled targets, as indicated in the figure legends. For testing the peptide specificity of CTL, effectors and ^{51}Cr-labeled targets were mixed with various concentrations of peptide at the beginning of the assay (19). The percentage specific ^{51}Cr release was calculated as 100(experimental release − spontaneous release)/(maximum release − spontaneous release). Maximum release was determined from supernatants of cells that were lysed by addition of 5% Triton X-100. Spontaneous release was determined from target cells incubated without added effector cells.

Peptide Synthesis. Synthetic peptides 13–23 residues long were prepared by the multiple simultaneous peptide method of solid-phase peptide synthesis, in polypropylene mesh "tea-bags" as described (31). Peptides were desalted by reverse-phase chromatography on C_{18} "Sep-Pak" columns (Waters), analyzed by HPLC, and prescreened for lack of nonspecific toxicity or mitogenicity (P. Hale, C. Ouyang, and J.A.B., unpublished observations).

RESULTS

Conditions for Eliciting Murine CTL Specific for HIV gp16 The ability of recombinant vaccinia viruses to prime ar stimulate CTL specific for the products of inserted vir genes (30) was used to generate CTL specific for HIV gp1 from BALB/c (H-2^d) mice. Specificity for gp160 was foun at three levels—lymphocyte priming, restimulation, an effector function (Table 1). Thus, only the recombina vaccinia virus expressing gp160 (vSC-25), not the contr vaccinia virus (vSC-8), could prime mice for development CTL able to kill the gp160-expressing fibroblast, 15-1 (compare groups 2 and 4 and groups 5 and 6). Likewise, onl syngeneic vSC-25-infected cells, or the gp160-expressin transfectant 15-12, but not cells infected with control viru vSC-8, could restimulate immune T cells *in vitro* to kill th specific target (15-12) (groups 4 and 6 vs. group 3). CTL fro gp-160-primed and restimulated spleen cells preferentiall killed gp160-expressing targets vs. control H-2 matche targets (groups 4 and 6). The modest nonspecific killing c control targets (18 neo) by vSC-25-restimulated spleen cell was absent when restimulation was done with the transfec tant rather than vaccinia-infected cells. Vaccinia (vSC-25) infected targets served as a positive control. Finally, long-term line of CTL effectors specific for gp160-expressin targets was established by repetitive stimulation of splee cells from vSC-25-immunized mice with 15-12 transfectant and Con A supernatant (group 7).

Identification of an Immunodominant CTL Epitope o gp160. A series of 41 overlapping peptides in 17 cluster covering 46% of the HIV gp160 env protein sequence, mostl

Table 1. Priming and boosting requirements for CTL induction and long-term CTL line production in H-2^d mice

Immunization	Restimulation	E/T ratio	% specific lysis on 18-*neo*	% specific lysis on 15-12	% specific lysis on 15-12/vSC-2
vSC-8	vSC-8	40:1	6.3	2.3	70.9
		13:1	3.7	−2.6	58.3
		4:1	1.6	−4.2	42.3
vSC-8	vSC-25	40:1	15.1	27.3	65.8
		13:1	6.3	8.4	59.2
		4:1	3.3	1.0	47.1
vSC-25	vSC-8	40:1	8.0	1.1	73.9
		13:1	3.8	−0.9	58.3
		4:1	2.1	−2.9	41.1
vSC-25	vSC-25	40:1	26.1	49.3	66.2
		13:1	11.4	25.5	56.9
		4:1	4.2	12.1	49.0
vSC-8	15-12	40:1	0.8	−2.8	11.4
		13:1	2.6	−1.6	9.0
		4:1	1.8	−1.3	5.2
vSC-25	15-12	40:1	6.2	55.9	54.4
		13:1	3.2	38.9	46.3
		4:1	2.1	24.9	3.1
vSC-25	15-12*	40:1	15.0	44.0	NT
		20:1	4.5	39.1	NT
		10:1	5.5	33.5	NT
		5:1	2.1	33.2	NT

BALB/c (H-2^d) mice were primed i.v. with 10^7 plaque-forming units of recombinant vaccinia virus expressing the HIV env protein gp160 (vSC-25) or the bacterial *lacZ* gene (vSC-8). The spleen cells were restimulated *in vitro* either with recombinant vaccinia virus (vSC-25 or vSC-8)-infected syngeneic spleen cells (2.5×10^6 cells per ml) or with gp160 gene-transfected BALB/c 3T3 cells (15-12) (2×10^5 cells per ml). CTL activity was measured against *neo* gene-transfected 3T3 cells (18-*neo*), 15-12, and vSC-25-infected 15-12 target cells (15-12/vSC-25). NT, not tested; E/T, effector/target cell ratio.

*Long-term CTL line produced by repetitive stimulation of vSC-25 immune spleen cells with transfectant 15-12 plus lymphokines.

selected by use of the amphipathicity algorithm for searching for immunodominant helper T-cell epitopes (17), was synthesized. To identify the T-cell epitopes recognized by gp160-specific BALB/c CTL, various concentrations of these peptides were individually added to effectors and ^{51}Cr-labeled fibroblast tumor targets at the start of the assay culture. We measured the cytotoxic activity of three types of effector cells: vSC-25 immune spleen cells stimulated once *in vitro* either with the 15-12 transfectant or with vSC-25-infected autologous cells (presumably polyclonal effector populations), and the long-term CTL line (possibly an oligoclonal population). The results show that among the various peptides tested, only one (no. 18) could sensitize H-2^d target cells for high levels of specific killing by all three types of effectors (Table 2). Therefore, we conclude that the sequence contained in peptide 18 (Arg-Ile-Gln-Arg-Gly-Pro-Gly-Arg-Ala-Phe-Val-Thr-Ile-Gly-Lys) contains an immunodominant epitope of gp160 for cytotoxic cells of the *H-2^d* haplotype. Some other peptides (nos. 21, 41, 42, and 47) appeared to marginally sensitize target cells, but these effects were very small compared to the activity of peptide 18. Killing specific for peptide 18 was effective at surprisingly low concentrations of peptide. At 0.1 μM peptide, the killing was still on a plateau, and only at 0.01 μM peptide did the killing fall to half-maximal (data not shown). All of the active peptides were identified as capable of forming an amphipathic helix.

Surface Phenotype and Class I MHC Restriction of the Anti-gp160 CTL. Treatment of the CTL effector cells with anti-Lyt2 monoclonal antibody plus rabbit complement, but not anti-L3T4 antibody plus complement or complement alone, led to loss of killing activity on either the 15-12 fibroblasts transfected with the gp160 gene or the control fibroblasts incubated in the presence of peptide 18 (Fig. 1). These data show that the effector cells that recognize and kill HIV envelope gp160 protein-expressing cells in our system, including those specific for peptide 18, are conventional Lyt2$^+$ L3T4$^-$ (CD8$^+$ CD4$^-$) CTL.

The fibroblasts used as targets in these experiments express class I but not class II MHC gene products. Therefore, the gp160-specific CTL capable of lysing the 15-12 transfectants or the peptide-bearing fibroblasts were likely to be class I MHC molecule-restricted, as is usual for Lyt2$^+$ effector T cells. Using two L-cell (H-2^k) transfectants, T1.1.1 and T4.8.3, expressing L^d or D^d, respectively (26, 27), we demonstrated that recognition of peptide 18 is restricted by the class I D^d molecule, not the L^d molecule (Fig. 2).

H-2^k Mice Appear to be Low Responders for Cytotoxic T Cells to gp160. We investigated a second haplotype, *H-2^k*, using the same protocol as in the case of *H-2^d*. L cells (H-2^k) were similarly transfected and shown to express gp160 mRNA in levels comparable to the 15-12 cell line. No cytotoxic activity could be detected against these targets using spleen cells from vSC-25 immune BALB.K (H-2^k) mice stimulated *in vitro* with syngeneic vSC-25-infected spleen cells or with the L-cell transfectant itself (Table 3). Similarly, none of the peptides tested produced targets that could be killed by such effector cells (Table 2). Despite the lack of gp160-specific CTL activity, the BALB.K mice were effectively primed and restimulated as they produced potent vaccinia-specific CTL (Table 3, from the same experiment as Table 2). Similar results were obtained with C3H (H-2^k) mice (Table 3 and data not shown). Because BALB.K mice are congenic to BALB/c responder mice but bear the *H-2^k* haplotype, the difference in responsiveness is MHC-linked. These results suggest that *H-2^k* is a CTL low responder to gp160 as assayed under the present conditions, in contrast to *H-2^d*, which is a high responder haplotype.

Table 2. Identification of target epitopes in gp160 in H-2^d and H-2^k mice

Peptide (sequence position)	% specific ^{51}Cr release BALB/c (H-2^d) V25/15-12 (40:1)	Line (10:1)	V25/V25 (40:1)	BALB.K (H-2^k) V25/V25 (40:1)
1 (93–107)	4.0	1.8	1.7	1.0
2 (98–112)	3.3	4.5	2.6	0.9
3 (102–116)	5.7	5.0	0.6	0.8
4 (103–117)	1.8	0.0	−0.6	0.4
5 (105–117)	0.5	0.5	3.6	−0.2
7 (107–121)	1.2	−1.1*	2.6	0.6
8 (112–127)	5.1	3.3	7.5	0.2
9 (141–155)	4.7	0.4	−2.2	0.1
10 (157–171)	−1.7	−6.5*	0.5	0.6
11 (231–245)	4.4	1.4	2.5	0.3
12 (236–250)	2.0	3.3	4.8	1.6
14 (252–273)	2.0	0.4	−1.6	1.2
17 (267–282)	5.8	4.5	4.1	−0.4
18 (308–322)	**67.6**	**56.4**	**52.5**	1.6
19 (317–331)	−3.0	−0.6*	−2.2	0.9
20 (335–349)	6.4	0.3	5.3	0.0
21 (343–357)	**10.8**	**8.4***	**14.1**	0.5
22 (354–368)	−0.1	−3.5	−0.6	0.7
23 (362–376)	0.9	5.9*	0.5	0.7
26 (421–436)	−2.8	−4.4	1.6	1.1
28 (425–439)	2.6	−1.2*	−0.1	0.8
29 (430–444)	3.2	1.6	0.2	1.0
30 (476–490)	5.2	3.9*	−1.0	0.8
33 (485–499)	6.0	1.9	−0.4	0.7
35 (553–574)	−3.1	−5.3*	−1.2	0.8
36 (612–626)	2.1	10.1*	−3.0	1.0
37 (619–633)	1.7	8.9	0.6	2.1
39 (629–643)	7.7	4.8*	0.4	0.6
40 (632–646)	−0.5	−0.4	−1.9	0.2
41 (637–651)	**7.9**	**9.7***	0.7	2.2
42 (657–671)	**12.0**	**6.3**	**7.0**	2.2
43 (662–676)	2.3	−0.1	−0.2	1.7
45 (723–737)	−0.8	−0.1	0.9	1.5
47 (780–794)	**9.9**	**10.5***	−1.5	1.1
50 (794–808)	−4.7	1.8*	−0.6	0.4
51 (799–813)	1.2	2.6	0.1	0.9
52 (821–835)	3.9	0.8	1.9	0.6
53 (827–841)	0.5	3.4*	−2.0	−0.1
55 (834–848)	3.1	4.4*	−4.4	0.0
56 (839–853)	2.8	1.9	−3.6	−0.8
57 (842–856)	0.6	−1.2*	−1.4	0.1
None	1.7	3.0	3.4	0.8

As targets, 18-*neo* transfectants (H-2^d) plus 10 μM synthetic peptide were used for BALB/c effectors and L28 (H-2^k) plus 10 μM peptide were used for BALB.K effectors. The ratios in parentheses are the effector/target cell ratios. V25/15-12 indicates effector cells derived from vSC-25-immunized BALB/c spleen cells restimulated *in vitro* with the 15-12 transfectant (see Table 1). Line indicates the CTL line derived from vSC-25-immunized BALB/c spleen cells repetitively stimulated with 15-12 and 10% Con A supernatant. V25/V25 indicates effector cells derived from vSC-25 immune spleen cells restimulated *in vitro* with vSC-25-infected syngeneic spleen cells. Controls for BALB.K cells from the same experiment are described in Table 3. Boldface indicates positive peptides in BALB/c. The National Biomedical Research Foundation sequence numbers shown are 7 less than those of Ratner *et al.* (32).
*Results were obtained at an effector/target cell ratio of 20:1 instead of 10:1. No peptide control was 3.0% for both ratios.

DISCUSSION

We have produced murine CTL with specific lytic activity for syngeneic cells expressing the HIV gp160 env glycoprotein. Elicitation of such HIV-specific CTL was achieved in the

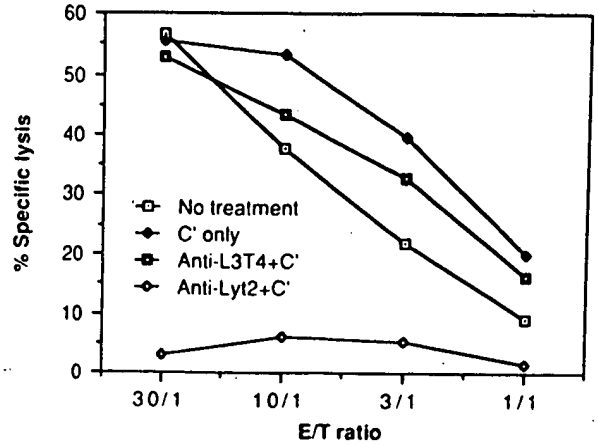

FIG. 1. Phenotype of the H-2^d CTL line specific for peptide 18-bearing cells. Five × 10^3 ^{51}Cr-labeled BALB/c 3T3 *neo* gene transfectants were cultured with cells from the long-term anti-gp160 CTL line at several effector/target cell ratios in the presence of 1 μM peptide 18. The effector cells were pretreated with monoclonal antibodies anti-L3T4 plus complement, anti-Lyt2 plus complement, or with complement only. Control group was untreated. E/T, effector/target cell.

H-2^d but not the *H-2^k* haplotype, suggesting that mice of these two haplotypes are gp160 immune response (*Ir*) gene high and low responders, respectively, under these experimental conditions. Because previous studies have shown that both haplotypes make class II helper T responses to gp160 (18), and the development of vaccinia-specific CTL by H-2^k mice indicates the presence of effective vaccinia-specific T-cell help, we suggest that the immune response defect in H-2^k is associated with class I MHC molecule-restricted T-cell responses.

Based on the recent observation that class I MHC molecule-restricted CTL recognize short peptide analogues of the original protein immunogen (19–21), we have analyzed the specificity of the CTL produced against HIV gp160 in the responder *H-2^d* haplotype. These experiments revealed a single peptide sequence corresponding to residues 308–322 (National Biomedical Research Foundation numbering) of the sequence of Ratner *et al.* (32) that accounted for virtually

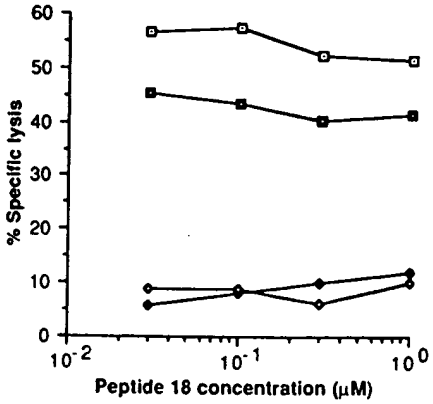

FIG. 2. CTL specific for peptide 18 are restricted by the class I molecule D^d. Five × 10^4 effector cells from the long-term line were cultured with 5 × 10^3 ^{51}Cr-labeled targets in the presence of various concentrations of peptide 18. T1.1.1 (26) (◆) and T4.8.3 (27) (■) indicate L-cell (H-2^k) transfectants expressing L^d and D^d, respectively. *neo* gene transfected 3T3 fibroblasts, 18-*neo* (K^d,L^d,D^d) (□) were used as a positive control and *neo* gene-transfected L-cells L28 (K^k,D^k) (◆) were used as a negative target control. Specific lysis in the absence of peptide was −0.2%, 0.3%, 1.5%, and 0.02% for the four targets, respectively. Spontaneous release ranged from 14.6% to 16.6%.

Table 3. H-2^k (BALB.K and C3H) mice fail to produce CTL specific for transfectants expressing the HIV envelope protein

Immunization	Restimulation	E/T	% ^{51}Cr release			
			25-20	L28	25-20/V25	L28/V25
vSC-25 (BALB.K)	vSC-25 inf.	80:1	1.2	0.5	56.2	57.7
		40:1	1.3	0.8	47.3	44.4
		20:1	0.7	0.6	39.4	39.0
vSC-25 (C3H)	vSC-25 inf.	80:1	3.7	1.7	NT	60.1
		40:1	1.9	0.4	NT	51.9
		20:1	−0.1	0.1	NT	41.5
vSC-25 (C3H)	25-20	60:1	11.8	15.7	NT	35.7
		20:1	6.8	8.5	NT	16.7
		7:1	3.9	2.3	NT	5.6

BALB.K (H-2^k) or C3H (H-2^k) spleen cells immunized with vSC-25 were restimulated *in vitro* with either the gp160-expressing L-cell transfectant 25-20 or vSC-25-infected (inf.) syngeneic spleen cells. After culture for 6 days, cytolytic activity of the restimulated cells was measured against 25-20, or *neo* gene-transfected L cells (H-2^k), L28, as a control target or vSC-25-infected 25-20 (25-20/V25) and L28 (L28/V25), as positive control targets. E/T, effector/target cell ratio; NT, not tested.

all the CTL activity in both polyclonal secondary CTL populations and in a long-term CTL line made with stimulator cells expressing the entire gp160 protein. Although we cannot exclude the existence of other epitopes corresponding to peptides we have not synthesized, and other epitopes will likely be found with other MHC haplotypes, preliminary cold-target inhibition experiments using peptide 18 and radiolabeled gp160-transfected cells confirm that virtually all H-2^d CTL generated under our culture conditions are specific for this single epitope (unpublished observations). The presence of only a single (immunodominant) epitope in the responder *H-2^d* haplotype and the lack of response in *H-2^k* mice indicate that the number of strong epitopes on the gp160 molecule for CTL recognition in mice in general may be extremely limited.

The peptide-dependent killing observed is mediated by conventional Lyt2$^+$ L3T4$^-$ CTL restricted to the D^d class I MHC molecule. The highly specific T-cell recognition of target cells incubated with synthetic peptide strongly supports the emerging hypothesis (19–21, 33) that class I- as well as class II-restricted T cells recognize degraded or denatured antigenic peptide fragments produced by active intracellular "processing." Because the concentration required for half-maximal killing was ≈10^{-8} M, we conclude that either peptide 18 binds with relatively high affinity to D^d, or the fraction of class I molecules on a cell surface that must be occupied by peptide to get effective killing is relatively low. The peptide 18-defined immunodominant epitope for class I-restricted CTL recognition has a sequence able to fold in an amphipathic helical secondary structure, like that we have found to be a good predictor of T-cell sites recognized by class II-restricted helper or proliferating T cells (15–18). Although we did not select the panel of peptides randomly to allow for a statistical test of the predictive method, this case adds to the small but growing list of sites seen by class I-restricted cytotoxic T cells that all have this property (34) and so suggests the possibility that the same type of peptide structures may be seen by both classes of T cells. Thus, the difference between class I- and class II-restricted T-cell recognition may depend not so much on the structure of the epitope itself, but on the pathway through which the antigen molecule is processed (33, 35).

The immunodominant site for CTL in H-2^d mice, residues 308–322 of gp120, overlaps the major B-cell epitope of this protein evoking virus-neutralizing antibody responses in humans and animals (36, 37). This epitope is highly variable in sequence among different isolates of HIV. Such variabil-

ity may result from selection of spontaneous mutant viral genomes encoding proteins altered so as to avoid targeting by the immune system. If such is the case, we cannot at this point determine whether the greater pressure comes from cytotoxic T cells or neutralizing antibodies, because the same site is seen by both. However, if part or all of the immune pressure does indeed come from CTL rather than antibody, it would suggest that the same peptide 18 site is immunodominant for CTL recognition in humans as well as mice and that CTL may be important in immunity against HIV. Recent reports of human anti-HIV CTL by Walker *et al.* (13) and Plata *et al.* (14) demonstrate that such responses occur in naturally infected individuals, and it has been suggested that the loss of CTL responses correlates with development of (the more aggressive stages of) AIDS. Moreover, at least for helper sites, there is precedent for the same epitopes being immunodominant in mice and humans (38–40).

To date, the predominant HIV vaccine strategy has focused on neutralizing antibodies more than cell-mediated immunity, although the latter effector mechanisms may be more suited to combating cell-to-cell virus transmission in this infection. The present identification of the first known CTL epitope on an HIV protein provides useful, yet disturbing, information for vaccine development. In particular, the location of an immunodominant CTL epitope at a site of interisolate variation presents the same problem as for type-specific neutralizing antibodies, although because CTL from many infected humans lysed target cells prepared with vSC-25 (13), at least some conserved epitopes may exist. A vaccine effective at stimulating CTL or humoral immunity may thus have to be polyvalent and consist of a mixture of recombinant proteins or synthetic peptides representing selected sequences from the variable regions of a number of viral isolates.

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